academicJournals

Vol. 7(10), pp. 552-559, 15 March, 2013
DOI 10.5897/AJPP12.1058
ISSN 1996-0816 © 2013 Academic Journals
http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

Full Length Research Paper

Penta-O-galloyl-beta-D-glucose enhances antitumor activity of imatinib and suppresses the growth of K562 cells in mice

Tae-Rin Kwon, Min ho Lee, Soo Jin Jeong, Eun Jung Sohn, Duckgu Lee, Eun Ah Shin, Hyo-Jeong Lee, Eun-Ok Lee, Ji Hoon Jung, Ji-Hyun Kim, Deok-Beom Jung and Sung-Hoon Kim*

College of Oriental Medicine, Kyung Hee University, Seoul 130-701, Republic of Korea.

Accepted 5 February, 2013

This study involves the antitumor potential of 1,2,3,4,6-penta-O-galloyl-beta-D-glucose (PGG) to induce apoptosis and enhance antitumor activity of imatinib in K562 cells. Though PGG was reported to have antitumor activities in breast, prostate, kidney, liver cancers and HL-60 leukemia cells, there is no report on synergistic antitumor effect of PGG with imatinib until now. In the present study, PGG significantly enhanced the cytotoxicty and cleavage of poly (ADP-ribose) polymerase (PARP) in imatinib induced chronic myeloid leukemia K562 cells. Furthermore, oral administration of PGG or imatinib significantly inhibited the growth of K562 cells inoculated in Balb/c athymic nude mice and also immunohistochemistry revealed decreased expression of Ki67 (proliferation), CD34 (blood density) and death domain-associated protein (DAXX) and increased terminal deoxynucleotide transferasemediated dUTP nick end labeling (TUNEL) positive cells as one of the apoptotic feature in tumor sections of K562 mouse xenograft model comparable to imatinib treated group. Overall, our findings suggest the potency of PGG to induce apoptosis and enhance antitumor activity of imatinib in K562 cells.

Key words: Chronic myeloid leukemia, apoptosis, 1,2,3,4,6-penta-O-galloyl-beta-D-glucose (PGG), imatinib.

INTRODUCTION

Chronic myelogenous leukemia (CML) is a myeloproliferative cancer with *BCR-ABL* fusion genes in hematopoietic progenitor cells. Bcr-Abl selective tyrosine kinase inhibitor imatinib mesylate (Glivec or Gleevec) has substantially improved the treatment landscape for CML (Druker et al., 1996). Nonetheless, some patients are refractory to imatinib and eventually develop imatinib resistance (Hochhaus et al., 2007). The resistance to imatinib is related with *BCR-ABL* dependent mechanism via point mutations on *Bcr-ABL* fusion protein or independent mechanism via activation of Src family kinase (Valent, 2007; Ramirez and DiPersio, 2008).

1,2,3,4,6-penta-O-galloyl-beta-D-glucose (PGG), a naturally occurring gallotannin polyphenolic compound from *Rhus chinensis* Mill, showed anti-proliferative, anti-angiogenic, anti-diabetic and apoptotic activities (Zhang et al., 2009).

Also, it is of note that PGG exerts antitumor activity in various cancers such as prostate (Hu et al., 2008), lung (Huh et al., 2005), breast (Chen et al., 2003), melanoma (Ho et al., 2002), liver cancers (Oh et al., 2001) and sarcomas (Miyamoto et al., 1987). Nevertheless, there are no evidences regarding the synergistic antitumor effect of PGG with imatinib so far. Thus, in the current study, the antitumor potential of PGG to induce apoptosis and enhance antitumor activity of imatinib in K562 cells and animal study using immunohistochemistry was elucidated *in vitro* and *in vivo*.

*Corresponding author. E-mail: sungkim7@khu.ac.kr. Tel: 82-2-961-9233. Fax: 82-2-964-1064.

MATERIALS AND METHODS

Isolation of PGG

PGG was isolated from the gallnut of *Rhus chinensis* Mill as previously described (Chai et al., 2010). The yellowish active compound was identified as PGG by nuclear magnetic resonance (NMR) and fast atom bombardment mass spectrometry (FAB-MS) analyses. The purity of PGG was estimated to be >96% by high performance liquid chromatography (HPLC).

Cell culture

K562 (CML) cells purchased from American Type Culture Collection (ATCC) (Rockville, MD) were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% fetal bovine serum (FBS).

Cell viability assay

Cytotoxicity of PGG was determined using sodium 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt (XTT) assay. Cells (2×10^4 cells/100 µl/well) were seeded on 96-well microplates, treated with various concentrations of PGG (0, 10, 20, 40 µM) for 24 h and incubated with XTT labeling mixture [125 µM XTT/25 µM phenazine methosulphate (PMS)] at 37°C for 2 h. Optical density (OD) was measured using a microplate reader (Molecular Devices Co., Sunnyvale, CA) at 450 nm. Cell viability was calculated as a percentage of viable cells in PGG-treated group compared with untreated control by following equation.

Cell viability (%) = [OD (PGG) − OD (Blank)] / [OD (Control) − OD (Blank)] × 100

Western blotting

Western blotting was performed as previously described (Seo et al., 2011). Whole cell extracts were prepared using cell lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM Ethylenediaminetetraacetic acid (EDTA), 1% NP-40, 0.25% deoxycholate acid) containing protease inhibitor cocktail (Roche Applied Science, Inndianapolis, IN). Protein samples were quantified by using Bio-Rad DC protein assay kit II (Bio-Rad, Hercules, CA), separated onto 8 to 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and electro-transferred to a nitrocellulose membranes. After blocking with 5% nonfat skim milk, the blots were probed with antibodies against Poly (ADP-ribose) polymerase (PARP) (Santa Cruz Biotechnology, Santa Cruz, CA), and β-actin (Sigma, St. Louis, MO), and exposed to horseradish peroxidase (HRP)-conjugated anti-mouse or rabbit secondary antibodies. Protein expression was detected by using enhanced chemiluminescence (ECL) system (Amersham Pharmacia, Piscataway, NJ).

Mouse xenograft model

The animal studies were conducted under guidelines approved by Institutional Animal Care and use Committee, Kyung Hee University [KHUASP(SE)-11-005]. Five-week-old female athymic nude mice were purchased from Jung Ang lab animal (Seoul, Korea) and maintained under conventional conditions. K562 cells (5×10^6 cells) were mixed with matrigel (Becton Dickinson, 50%, in 100 µl) and injected subcutaneously on the right flank of the mice. After 5 days of inoculation, the mice were given intraperitonial (i.p) injection of PGG at 4 or 10 mg/kg body weight or imatinib at 25 or 50 mg/kg body weight in 50% PEG 400/50% saline (v/v) every 2 days for 18 days. Control mice were administered the solvent vehicle. Tumor volume was measured every other day with caliper and calculated according to the formula; $V = 0.52a^2b$, where *a* is the smallest superficial diameter and *b* is the largest superficial diameter.

Immunohistochemistry

The animals were sacrificed 18 days after inoculation of K562 cells. Tumors were immediately removed, fixed in 4% PFA, paraffin-embedded, and sectioned at 4 µm. Antigen retrieval was performed after dewaxing and dehydration of the tissue sections by microwaving them for 10 min in 10 mM citrate buffer. Sections were cooled to room temperature, treated with 3% hydrogen peroxide in methanol for 10 min, and blocked with 6% horse serum for 40 min at room temperature. Sections were then incubated with the primary antibodies for Ki-67 (Lab Vision Corporation, Fremont, CA), CD34 (Abcam, Boston, MA), TUNEL (Calbiochem, Darmstadt, Germany), and DAXX (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight. Sections were washed in PBS and incubated with secondary antibody (biotinylated goat anti-rabbit (Vector laboratories, Burlingame, CA) or biotinylated rabbit anti-rat IgG (Abcam, Boston, MA) for 30 min. The antibodies were detected with the vector avidin biotin complex (ABC)/HRP kit (Vector Laboratories, Burlingame, CA) and color-developed with 3,3'-diaminobenzidine tetrahydrochloride (DAB·4HCl).

Statistical analysis

Data were presented as means ± standard deviation (SD) of a minimum of 3 or more replicates. The statistically significant differences between control and PGG-treated groups were calculated by Student's *t*-test using Sigmaplot software (Systat Software Inc., San Jose, CA).

RESULTS

PGG synergistically enhances the antitumor activity of imatinib in K562 cells

Imatinib is an anti-cancer drug currently used to treat chronic myeloid leukaemia (CML) patients by targeting Bcr-Abl activity (Druker and Lydon, 2000). We tested the possibility that PGG could stimulate apoptosis in CML cells induced by Imatinib. Cells were treated with various concentrations of PGG (0, 10, 20 or 40 µM) in the absence or presence of imatinib (0.25 µM) for 24 h. The cell viability was significantly decreased in combination of PGG with imatinib in a dose-dependent manner compared with the cells treated with either drug alone (Figure 1A). PGG obviously revealed the synergistic effect on imatinib-induced cell death with combination interval (CI) value = 0.180, 0.005 and 0.001 at 10, 20 and 40 µM, respectively (Figure 1B). In addition, PGG treatment further increased PARP cleavage by combination with imatinib (Figure 1C). These results suggest the potential of PGG

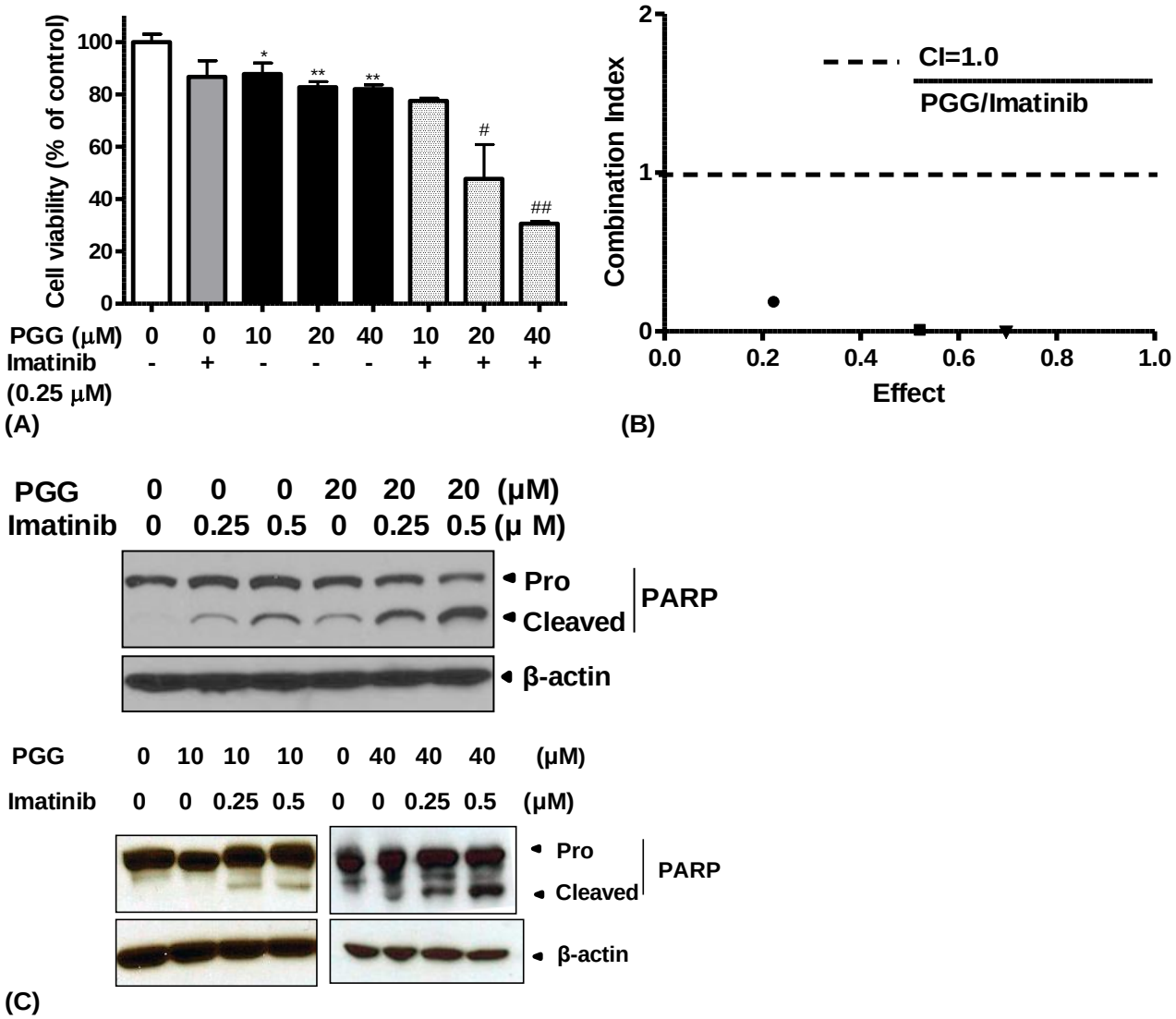

Figure 1. Combination of PGG and imatinib synergistically induces apoptosis in K562 cells. (A) Cells were treated with various concentrations of PGG (0, 10, 20 or 40 μM) and/or imatinib (0.25 μM) for 24 h. XTT assay was performed to measure the cytotoxicity of PGG and/or imatinib. Data are presented as means ± SD. *$p < 0.05$ and **$p < 0.01$ versus untreated control, and $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ versus imatinib control. (B) The combination index (CI) between PGG and imatinib was determined by Chou-Talalay method and CalcuSyn software (Biosoft, Ferhuson, MO). (C) Cells were treated with PGG (10, 20 or 40 μM) and/or imatinib (0, 0.25 or 0.5 μM) for 24 h. Cell lysates were prepared and subjected to Western blotting for PARP.

for combinational therapy suggest the potential of PGG for combinational therapy with imatinib for CML chemotherapy. Consistent with PARP cleavage, treatment of PGG (20 μM) with imatinib (0.25 μM) increased the accumulation of the sub-G1 apoptotic portion by 5.15 ± 0.34 compared to control (0.8 ± 0.08) or PGG alone (2.8 ± 0.08) (Figure 2).

PGG influences the expression of cancer biomarkers and DAXX in K562 mouse xenograft model

To verify the *in vivo* anti-tumor efficacy of PGG, K562 cells were subcutaneously injected into Balb/c athymic nude mice in the flank area, and starting after 5 days inoculation, the mice were intraperitoneally administered PGG (4 or 10 mg/kg) or imatinib (25 or 50 mg/kg) (a positive control) every 2 days. Tumor growth was monitored for 18 days. As shown in Figure 3, tumor size and weight in PGG- or imatinib-treated groups were decreased compared with untreated control without significant body weight loss. For analyzing biomarkers of anti-tumor efficiency, we conducted immunohistochemistry analysis with tumor sections from the mice (Figure 4).

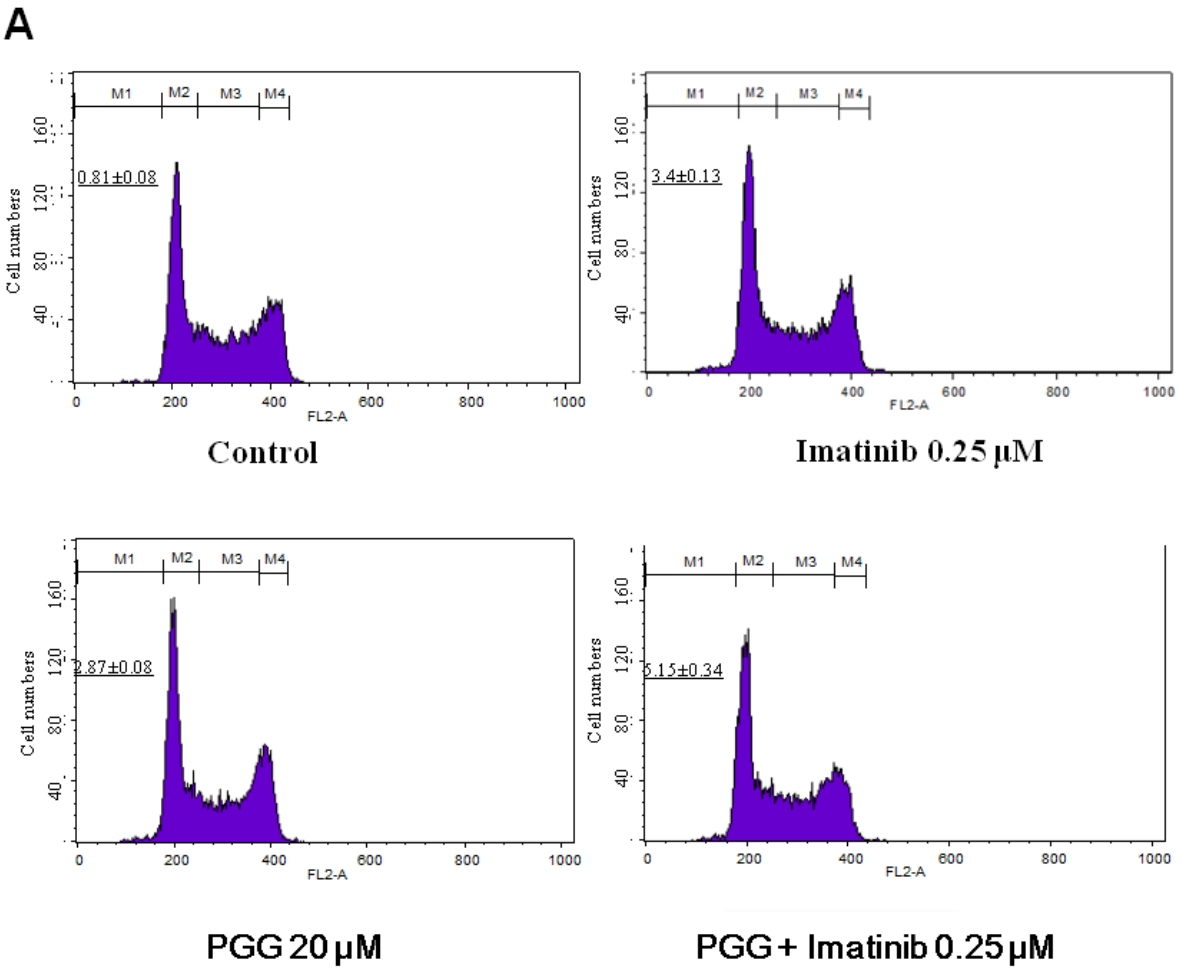

Figure 2. Combination of PGG and imatinib synergistically accumulates the sub-G1 population in K562 cells. (A) FACS analysis with propidium iodide (PI) staining. Cells were treated with PGG (20 μM) and/or imatinib (0.25 μM) for 24 h and stained with PI. (B) Bar graphs represent the percentages of sub-G1 DNA contents. Data represent means ± S.D. ***$p < 0.001$ versus untreated control, $^{\#\#}p < 0.01$ versus PGG alone.

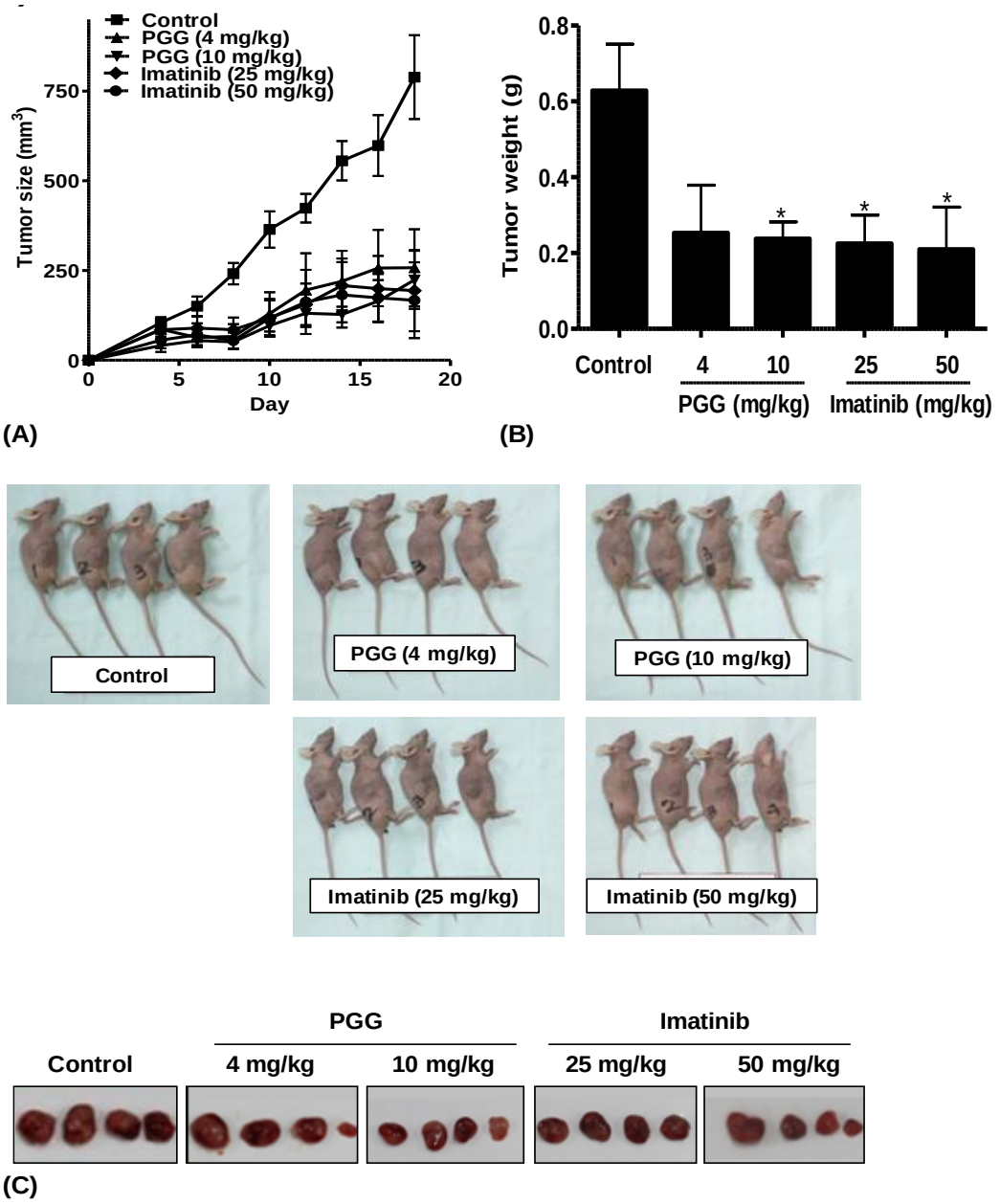

Figure 3. PGG suppresses K562 xenograft growth in female athymic nude mice. Five-week-old Balb/c nude mice were subcutaneously injected in the flank area with 5×10^6 cells in 100 µl of matrigel mixed with PBS. Five days after inoculation, mice (n = 4/group) were treated with vehicle (50% PEG400/50% saline (v/v)), PGG (4 or 10 mg/kg body weight) or imatinib (25 or 50 mg/kg body weight) by i.p. injection every 2 days for 18 days. (A) Tumor volume was calculated according to the formula $V = 0.25a^2b$, where *a* is the smallest superficial diameter and *b* is the largest superficial diameter. (B) Tumors were immediately removed and weighed at termination of experiment. Data are presented as means ± SD. *p < 0.05 versus untreated control. Photographs display selected tumor bearing mice and their dissected tumors.

The indicators of proliferation marker Ki-67 (first column) and vascular endothelial marker CD34 (second column) were significantly decreased in both PGG and imatinib-treated groups. Also, TUNEL positive stained cells were significantly increased in both PGG- and imatinib-treated mice (third column). Furthermore, positive cells for DAXX which are known as a proapoptotic protein were significantly reduced in PGG- and imatinib-treated

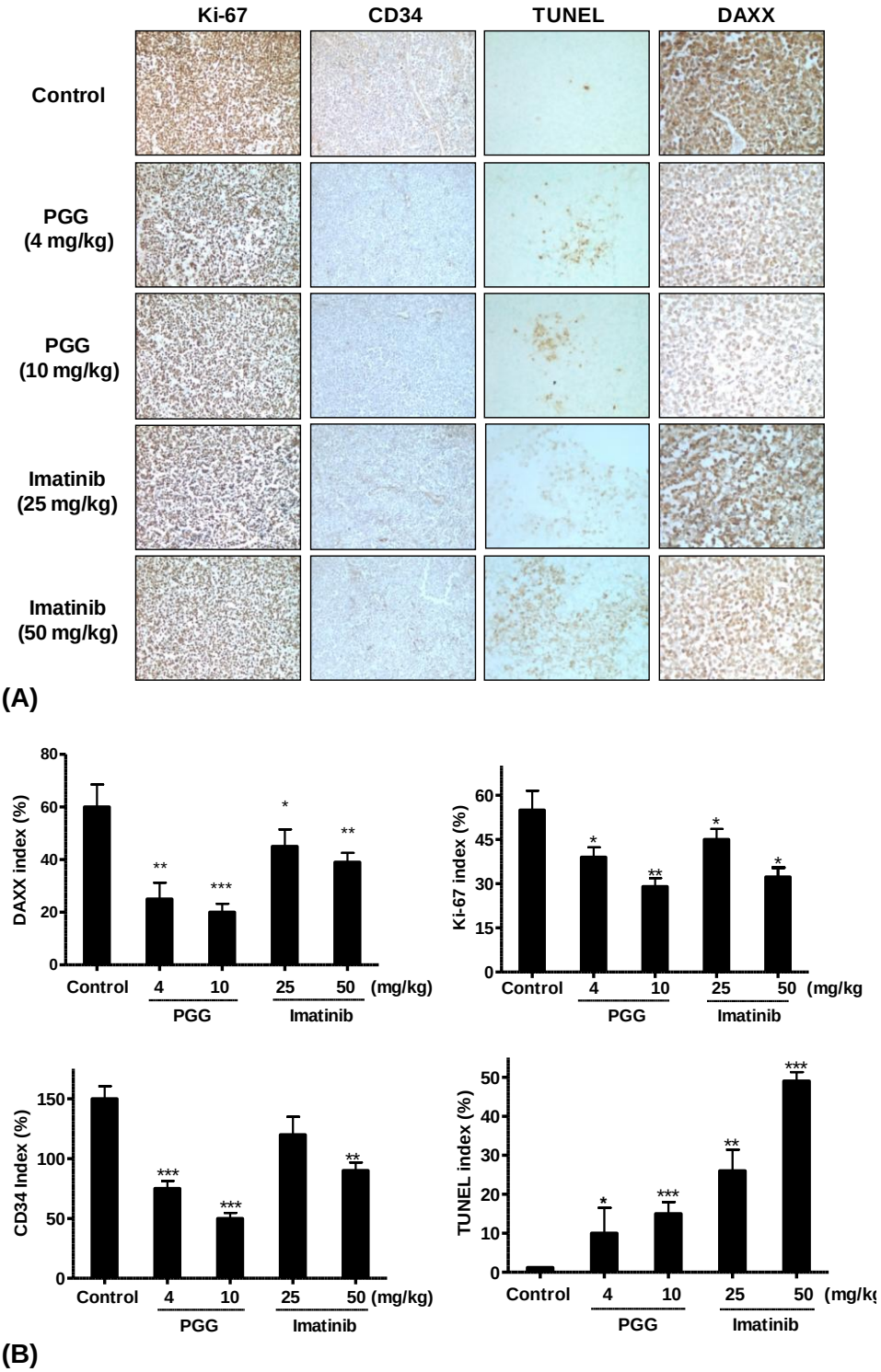

Figure 4. PGG exhibits anti-tumor activity in K562 mouse xenograft model. Mice were sacrificed on day 18 after cell inoculation, and tumors were immediately removed, fixed, embedded and sectioned at 4 μm for immunostaining of biomarkers Ki-67, CD34, TUNEL and DAXX at ×400 (Ki-67 and CD34) or ×200 (TUNEL and DAXX) of original magnification. The sections were detected as DAB substrate staining. Graphs show the Ki-67 index (proliferation), CD34 index (angiogenesis), TUNEL index (apoptosis) and DAXX index in tumor sections. Data are presented as means ± SD. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ versus untreated control.

mice (fourth column).

DISCUSSION

Several groups reported anti-cancer effects of natural products against CML through the reactive oxygen species (ROS) signaling. For instance, Mao et al. (2008) reported that shikonin induced apoptosis through the ROS/ c-Jun N-terminal kinases (JNK)-mediated process in CML cell lines K562 and LAMA84. Rakshit et al. (2010) reported the role of ROS in chlorogenic acid (Chi)-induced cell death in CML cell lines as well as primary leukemia cells from CML patients. In addition, Zhang et al. (2008) reported that beta-phenylethyl isothiocyanate (PEITC) exerted the cytotoxic effect in Glivec-resistant CML cells by regulating redox signaling. These evidences suggest the potential of natural products for CML treatment.

Combination cancer therapy has been thought as an effective process to increase the therapeutic efficiency of anti-cancer agents and reduce their adverse effects. Interestingly, PGG potentiated imatinib induced apoptosis as a combination therapy for CML with the value of CI > 1, determined by Chou-Talalay method and CalcuSyn software, implying significant synergism between PGG and imatinib. We recently found the protective effect of PGG on anti-cancer drug cisplatin-induced apoptosis in normal renal epithelial cells (data not shown), supporting the potential of PGG as a supplement of imatinib for a combination therapy for CML.

We finally found that PGG administration suppressed the growth of K562 xenograft without significant body weight loss. Immunohistochemistry showed decreased expression of Ki67 (proliferation), CD34 (angiogenesis), DAXX and increased TUNEL (apoptosis) positive cells in tumor sections of PGG treated mice, indicating the antitumor effects PGG were associated with anti-proliferation, anti-angiogenesis and apoptosis induction.

PGG suppressed the growth of K562 cells in xenograft nude mice and also immunohistochemistry confirmed antitumor effects PGG were associated with anti-proliferation, anti-angiogenesis and apoptosis induction. However, it still requires further study to confirm *in vitro* synergistic antitumor activity of PGG with imatinib in a large number of athymic nude mice and also elucidate molecular mechanism in another resistant CML cell lines in the near future.

Conclusion

PGG revealed the synergistic effect on imatinib-induced cell death in K562 cells *in vitro* and oral administration of PGG or imatinib significantly inhibited tumor size and weight of K562 cells inoculated in Balb/c athymic nude mice. Furthermore, immunohistochemistry assay revealed that the expressions of proliferation marker Ki-67 and vascular endothelial marker CD34 and (DAXX) were inhibited and also apoptosis marker TUNEL stained cells were significantly increased from the tumor tissues from PGG or imanitib treated groups. Overall, our findings suggest that PGG showed anticancer potential to induce apoptosis and enhance antitumor activity of imatinib in K562 CML cells.

ACKNOWLEDGEMENTS

This work was supported by the Korea Science and Engineering Foundation (KOSEF) grant funded by the Korea government (MEST) (No. 2012-0005755) and a grant from the Next-Generation BioGreen 21 Program (No. PJ007998), Rural Development Administration, Republic of Korea.

Abbreviations: CML, Chronic myelogenous leukemia; **DAXX,** death domain associated protein; **PGG,** 1,2,3,4,6-penta-O-galloyl-beta-D-glucose.

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